ARTHRITIS & RHEUMATISM
Vol. 56, No. 8, August 2007, pp 2503–2511
DOI 10.1002/art.22817
© 2007, American College of Rheumatology

Mutation and Citrullination Modifies Vimentin to a Novel Autoantigen for Rheumatoid Arthritis

Holger Bang,[1] Karl Egerer,[2] Anke Gauliard,[2] Kirsten Lüthke,[3] Paul E. Rudolph,[4]
Gert Fredenhagen,[1] Wigbert Berg,[1] Eugen Feist,[2] and Gerd-R. Burmester[2]

Objective. **Modification of antigens represents a trigger for the generation of autoantibodies. In the pathogenesis of rheumatoid arthritis (RA), citrullination of proteins has been shown to be a critical process, and the determination of antibodies against citrullinated antigens has been a diagnostic milestone. We undertook this study to determine whether antibodies to mutated and citrullinated vimentin (MCV) could serve as a diagnostic and prognostic marker for RA.**

Methods. **We identified novel isoforms of human MCV in the synovial fluid of RA patients. The significance of these disease-related modifications was investigated by the analysis of autoantibody reactivities. In a group of 1,151 RA patients, the diagnostic significance and the prognostic value of an anti-MCV enzyme-linked immunosorbent assay (ELISA) were compared with that of an anti–cyclic citrullinated peptide (anti-CCP) ELISA.**

Results. **In RA, sensitivities of 82% and 72% were calculated for the anti-MCV and anti-CCP assays, respectively. The specificity of both assays was comparable (98% and 96%, respectively). In followup analyses of 16 RA patients with moderate disease activity (mean Disease Activity Score in 28 joints [DAS28] of 2.72) and 26 RA patients with active disease (mean DAS28 of 5.07), disease stratification of RA was possible using the anti-MCV assay ($P = 0.0084$). A significant correlation of anti-MCV antibodies with the DAS28 was documented ($r = 0.5334$, $P = 0.0003$), in 42 RA patients ($n = 427$ antibody determinations at different time points).**

Conclusion. **Antigenic properties of vimentin were determined by mutation and citrullination. Anti-MCV antibodies are a novel diagnostic marker for RA. Furthermore, they may allow monitoring and—if confirmed in even larger series of patients—stratification of disease.**

Rheumatoid arthritis (RA) is a heterogeneous disease of multifactorial etiology and unpredictable outcome (1,2). Early diagnosis and treatment are essential in order to prevent erosive joint destruction, and therefore, efforts are being made to search for reliable diagnostic and prognostic markers. Recently, the diagnosis of RA was substantially improved by the introduction of standardized immunoassays for the detection of autoantibodies against citrullinated antigens (3–5). Despite the excellent performance of these immunoassays, anti–cyclic citrullinated peptide (anti-CCP) antibodies provide only a sensitivity comparable with that of rheumatoid factor (RF), and analysis of the correlation with RA disease activity yielded conflicting results (6,7). Moreover, this artificial antigen of a so-far-undisclosed composition is not expressed in the affected tissue and is therefore presumably not directly involved in the pathogenesis of RA.

Anti-Sa antibodies have been characterized as a specific marker for RA, including early disease manifestations (8–10). However, the sensitivity of anti-Sa was relatively low compared with that of RF. Immunoprecipitation experiments revealed that vimentin, an abundant structural component of the intermediate filaments, represents the target of anti-Sa antibodies (11). Importantly, the antigenic properties of vimentin were substantially triggered by citrullination, and further-

Supported by grants from the Dr.-Karl-Wilder-Stiftung, Berlin, and the National Genome Research Network (NGFN-2, SIPAGE) (Federal Ministry of Education and Research, Germany).

[1]Holger Bang, PhD, Gert Fredenhagen, Wigbert Berg, PhD: Orgentec Diagnostika, Mainz, Germany; [2]Karl Egerer, MD, PhD, Anke Gauliard, MD, Eugen Feist, MD, Gerd-R. Burmester, MD: Charité Universitätsmedizin Berlin, Berlin, Germany; [3]Kirsten Lüthke, MD: Lüthke/Boche, Dresden, Germany; [4]Paul E. Rudolph, PhD: Research Institute for the Biology of Farm Animals, Dummerstorf, Germany.

Address correspondence and reprint requests to Gerd-R. Burmester, MD, Department of Rheumatology and Clinical Immunology, Charité Universitätsmedizin Berlin, Schumannstrasse 20/21, 10117 Berlin, Germany. E-mail: gerd.burmester@charite.de.

Submitted for publication March 16, 2006; accepted in revised form May 4, 2007.

more, citrullinated peptides of vimentin were found to be presented via the HLA–DR4 shared epitope (12). Several other investigations linked vimentin by its known function, such as modification during apoptosis and secretion from macrophages in response to tumor necrosis factor α signaling, to the pathogenesis of RA (13–15). However, the antibody response against citrullinated vimentin shows a diagnostic sensitivity of only 40% for RA (16,17).

In the present study, modifications of vimentin were investigated under pathogenic conditions (e.g., oxidative stress) in more detail. A mutated isoform of vimentin was identified by mass spectroscopy analysis of purified vimentin from a human fibroblast cell line and from synovial fluid (SF) samples from RA patients. Subsequently, mutated vimentin was expressed and citrullinated for standardized detection of the respective antibodies in RA patients and in patients with rheumatic diseases other than RA by enzyme-linked immunosorbent assay (ELISA).

PATIENTS AND METHODS

Patients. We investigated 1,151 patients with RA fulfilling the 1987 revised criteria of the American College of Rheumatology (ACR; formerly, the American Rheumatism Association) (18), 189 patients with systemic lupus erythematosus (SLE) fulfilling the 1982 revised criteria of the ACR (19), 68 patients with primary Sjögren's syndrome (SS) diagnosed according to the criteria described by Vitali et al (20), and 232 healthy donors. Followup analyses (at least 10 consecutive blood samples in a 2–3-year time period) were performed in 16 patients with moderate RA (mean $\pm$ SD age 52.95 $\pm$ 13.15 years, male:female ratio 7:9, 10 RF positive, mean $\pm$ SD radiographic score 1.02 $\pm$ 0.34 on the Larsen scale [21]), 26 patients with active RA (age 52.65 $\pm$ 10.75 years, male:female ratio 7:19, 22 RF positive, Larsen score 2.75 $\pm$ 1.15), 10 patients with primary SS (age 46.3 $\pm$ 17.4 years, male:female ratio 0:10, 9 RF positive), and 10 patients with SLE (age 45.5 $\pm$ 16.6 years, male:female ratio 1:9, none of them RF positive). All samples were obtained from patients of the Department of Rheumatology and Clinical Immunology, Charité Universitätsmedizin Berlin after approval by the local ethics committees or from the Outpatient Department for Rheumatology Lüthke/Boche, Dresden. SF samples were obtained from affected knees of RA patients and were pooled.

Cell culture. The human fibroblast cell line WS1 (CRL-1502; American Type Culture Collection, Rockville, MD) was cultured for no longer than 8 passages in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum at 37°C in a humidified incubator with 5% CO_2. All experiments were done with cells between passages 3 and 4. Upon reaching a 90% confluence, cells were used for the vimentin characterization experiments.

To produce chronic oxidative stress, WS1 fibroblasts were passed at a density of 50,000/ml. After treatment with 1 μM H_2O_2, cells were returned to the incubator for 2 days, and proteins were extracted as described below. A stock solution of H_2O_2 (30% weight/weight) was diluted in phosphate buffered saline (PBS) for all cell treatments.

Semipurification of vimentin from fibroblasts. For 1 preparation, 1×10^9 fibroblasts were used from different cultures, harvested by trypsinization, snap-frozen in liquid nitrogen, and stored in liquid nitrogen until purification. Frozen fibroblasts were resuspended in 4 ml lysis buffer (200 m*M* Tris HCl, 8*M* urea, 1% Nonidet P40, 20 m*M* $CaCl_2$, 8 m*M* dithiothreitol [DTT], 2 m*M* EDTA, 20 m*M* methylammonium chloride, 40 m*M* 4-[2-aminoethyl]benzenesulfonyl fluoride [AEBSF], and 40 μg/ml of leupeptin and aprotinin, pH 7.4; Sigma, St. Louis, MO). After homogenization by sonication and centrifugation at 100,000*g* for 30 minutes at 4°C, the supernatant was collected and separated by gel filtration on Superdex 200 (HiLoad 16/60, prep grade; Amersham Biosciences, Freiburg, Germany), equilibrated with buffer G (50 m*M* Tris HCl, 2*M* urea, 5 m*M* $CaCl_2$, 2 m*M* DTT, 0.5 m*M* EDTA, 5 m*M* methylammonium chloride, 10 m*M* AEBSF, and 10 μg/ml of leupeptin and aprotinin, pH 7.4). Western blot analyses of the fractions obtained were performed with goat polyclonal antivimentin IgG antibodies (C-20, sc-7557; Santa Cruz Biotechnology, Santa Cruz, CA) as well as with serum samples from patients with RA and were visualized by using an ECL Western blotting detection kit (Amersham Biosciences).

Citrullinated proteins were detected as described by Senshu et al (22) by using a commercially available anticitrulline detection kit (Upstate Biotechnology, Lake Placid, NY) according to the manufacturer's procedures. A modification buffer was prepared by mixing 5 ml of reagent A (0.025% $FeCl_3$, 4.6*M* H_2SO_4, 3.0*M* H_3PO_4) and 5 ml of reagent B (1% diacetyl monoxime, 0.5% antipyrine, 1*M* acetic acid), adding the mixture to nitrocellulose blot in a light-proof container, and incubating the mixture overnight at 37°C without agitation. After rinsing the nitrocellulose in water, the modified nitrocellulose was blocked with 3% nonfat dry milk in Tris buffered saline. Anti–modified citrulline (diluted 1:1,000) was incubated overnight at 4°C with agitation. After washing, the immunoblot was incubated with a 1:5,000 dilution of goat anti-rabbit horseradish peroxidase (HRP)–conjugated IgG for 1 hour at room temperature with agitation. The signals of citrullinated protein were visualized by using an ECL Western blotting detection kit.

The identified vimentin-containing fractions were pooled and stored at -20°C until further purification. The above-described procedure was repeated several times to collect sufficient material. The vimentin pool was reinforced by ultrafiltration on a Vivaspin 30 instrument (Millipore, Eschborn, Germany), and the protein concentration was adjusted to 200 μg/ml for each run of isoelectric focusing (IEF). To prepare 1 ml of sample for IEF fractionation (ZOOM IEF Fractionator; Invitrogen, Karlsruhe, Germany), 0.78 ml IEF denaturant was added according to the recommendations of the manufacturer (Invitrogen). Immediately after IEF migration, the fractions were collected and frozen at –20°C. Samples of each IEF fraction containing $\sim$20 μg of protein were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) (4–12% NuPAGE; Invitrogen), transferred to nitrocellulose membranes, and analyzed by Western blotting using the procedures described above.

Preparation of vimentin from SF. To prepare vimentin for amino acid sequencing and mass spectroscopy, the above-established purification procedure was used, with the following modifications. Lysis buffer was added to the pooled SF samples without separation of the containing cell material. After sonification and centrifugation, ultrafiltration was performed with the Vivaspin 30. By size-exclusion chromatography, vimentin isoforms were eluted at ~60 kd in buffer G. Immunoblotting and IEF were performed as described above. IEF fractions were subsequently purified by reverse-phase high-performance liquid chromatography (RP-HPLC) on a C4 semipreparative column (Amersham Biosciences). Using a linear gradient from 0% to 60% acetonitrile in 0.1% trifluoroacetic acid, vimentin isoforms were eluted at 48% acetonitrile. Fractions containing the vimentin antigen were evaporated and sent for microsequencing to TopLab (Munich, Germany). Additionally, bioinformatic analysis of vimentin modifications and mutations was done by ProteoSys (Mainz, Germany).

Mass spectroscopy. Peptides generated by in-gel digestion with trypsin were separated by liquid chromatography followed by double mass spectrometry (MS/MS) and then sequenced by Edman degradation. Matrix-assisted laser desorption ionization–time-of-flight (MALDI-TOF) MS (Voyager-DE STR Biospectrometer; PerSeptive Biosystems, Framingham, MA) and MALDI-TOF/TOF MS (4700 Proteomics Discovery System; Applied Biosystems, Foster City, CA) were used for highly reproducible identification of proteins by peptide mass fingerprinting and peptide sequencing. Proteins were identified by correlation of uninterpreted tandem mass spectra to entries in Swiss-Prot/TrEMBL using ProteinLynx Global Server (version 1.1; Micromass, Manchester, UK). One missed cleavage per peptide was allowed, and the fragment ion mass tolerance window was set to 100 parts per million. All matching spectra were reviewed by an expert, and citrullinated residues were localized by manual interpretation of sequence-specific fragment ions with the MassLynx program PepSeq (Micromass). The peptide sequences obtained were used to identify the purified antigen using the National Center for Biotechnology Information nonredundant protein database.

Molecular cloning, expression, and purification of recombinant human vimentin. The complementary DNA (cDNA) fragment encoding amino acids 1–465 of human vimentin was amplified from a vimentin full-length cDNA clone (primary accession no. P08670; OriGene, Rockville, MD) by polymerase chain reaction using a plasmid template and primers containing restriction sites for pET14b (tagged with His_6) (Novagen, Madison, WI) according to the recently reported procedure for α-fodrin (23). For mutation of vimentin at positions 16 and 59, a site-directed mutagenesis kit from Stratagene (La Jolla, CA) was used to insert a glycine instead of an arginine amino acid residue. Additionally, the reported polymorphism of arginine to histidine at position 50 was included.

Restriction digestion and DNA sequencing verified the final clones. The recombinant plasmid containing the vimentin gene was transformed to *Escherichia coli* BL21(DE3)pLysS. Expression of His_6-vimentin was induced by adding IPTG at 37°C. Harvested cells were resuspended in buffer (50 m*M* Tris HCl, 1 m*M* EDTA, 300 m*M* NaCl, 5% glycerol, 1 m*M* DTT, pH 8.0) and exposed to lysozyme treatment. Inclusion bodies

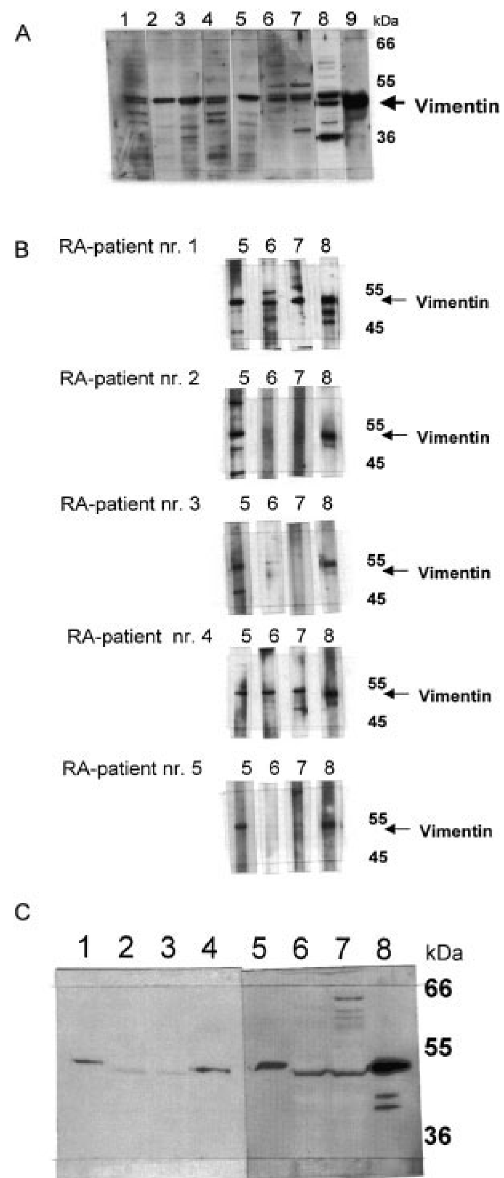

Figure 1. A, Semipurified vimentin isoforms derived from untreated fibroblasts (lanes 1–4) or fibroblasts treated with H_2O_2 (lanes 5–8) were immunoblotted using a goat antivimentin antibody. Vimentin isoforms are detectable at pI 3.0–4.6 (lanes 1 and 5), 4.6–5.4 (lanes 2 and 6), 5.4–6.2 (lanes 3 and 7), and 6.2–7.0 (lanes 4 and 8). Human recombinant vimentin served as a positive control (lane 9). **B,** Immunoblots from identical chromatofocusing fractions with vimentin semipurified from treated fibroblasts were prepared, sliced into strips, and analyzed with representative sera from 5 patients with rheumatoid arthritis (RA). Arrows indicate isoforms of vimentin. **C,** Immunoblots prepared as in **A** were analyzed with Senshu antibodies against chemically modified citrulline residues.

were dissolved by addition of a urea buffer (10 m*M* Tris HCl, 8*M* urea, 150 m*M* NaCl, pH 8.0) and applied to an Ni^{2+}-NTA Superflow column (Qiagen, Hilden, Germany) equilibrated

with buffer A (10 m*M* Tris HCl, 8*M* urea, 5 m*M* methylammonium chloride, 300 m*M* NaCl, pH 7.4). Nonspecifically bound proteins were removed by washing with buffer A, and vimentin was eluted with 100 m*M* imidazole in buffer A. Finally, the recombinant vimentin was purified on an ANX-Sepharose (Amersham Biosciences) ion-exchange column with a linear 0–1*M* NaCl gradient in buffer B (50 m*M* Tris HCl, 5 m*M* $CaCl_2$, 2 m*M* DTT, 0.5 m*M* EDTA, 5 m*M* methylammonium chloride, pH 7.4). Recombinant vimentin was homogeneous, as shown on Coomassie blue–stained SDS–polyacrylamide gels.

Citrullination of vimentin. Human recombinant vimentin and its mutated isoforms were citrullinated in vitro by rabbit muscle peptidyl arginine deiminase (PAD; Sigma) (40 units of PAD per milligram of vimentin) for 3 hours at 55°C in a buffer containing 50 m*M* Tris HCl, 5 m*M* $CaCl_2$, 2 m*M* DTT, 0.5 m*M* EDTA, 5 m*M* methylammonium chloride, pH 7.4. The reaction was stopped by adding EGTA (pH 8.0) to a final concentration of 50 m*M*. The extent of the citrullination was estimated using a MALDI-TOF Bruker Reflex mass spectrometer (TopLab).

Detection of antibodies to mutated and citrullinated vimentin (MCV) by ELISA. In ELISA, microtiter plates (Costar, Cambridge, MA) were incubated overnight at 4°C with purified MCV (0.5 μg/ml) diluted in PBS. Nonspecific antibody binding was blocked by PBS containing 1% bovine serum albumin and 0.02% NaN_3. Patient sera were diluted 1:100 in PBS–0.1% Tween 20 (pH 7.2) and incubated for 30 minutes. As a secondary antibody, an HRP-labeled goat anti-human (IgG- or IgA-specific) antibody (Dianova, Hamburg, Germany) was added for 15 minutes. Bound antibodies were visualized with tetramethylbenzidine solution. Absorbance was measured at 450 nm using an ELISA reader (Sunrise Reader; Tecan, Crailsheim, Germany). Standard curves were established by using a patient serum. The cutoff (25 units/ml) was defined as the mean + 3 SD antibody reactivity in sera from 232 blood donors. The lower limit of detection for anti-MCV IgG antibodies was determined to be 1.0 units/ml.

Detection of anti-CCP antibodies and RF by ELISA. For the detection of anti-CCP antibodies, a commercially available second-generation ELISA (Euro-Diagnostica, Malmö, Sweden) was used following standard procedures. RFs (IgG, IgM, and IgA) were determined with commercial ELISAs (Orgentec Diagnostika, Mainz, Germany).

Statistical analysis. Means, SDs, coefficients of variation (CVs), and *t*-tests were calculated by SAS procedures MEANS and TTEST (SAS system, release 9.1.3; SAS Institute, Cary, NC). The correlation analyses were performed by SAS procedure CORR (Base SAS 9.1.3 Procedures Guide, 2004; SAS Institute).

RESULTS

Identification of vimentin isoforms generated by oxidative stress. In initial studies, vimentin and the Sa antigen were purified from soluble cell extracts using ion-exchange chromatography. However, this procedure has limitations in detecting unknown structures or isoforms of vimentin. To disclose alternative modifications of vimentin, semipurification of the antigen was performed under denaturing conditions by size-exclusion chromatography as the initial approach. Preparative chromatofocusing revealed vimentin with a different pI in addition to the former known isoforms (results not shown).

To investigate whether these isoforms were induced by spontaneous DNA damage (e.g., under the influence of free radicals of oxygen), fibroblast cell cultures were exposed to oxygen stress. Experimentally, chronic oxygen influence was mimicked by low doses of H_2O_2 (n*M* to 10 μM concentration range; incubation

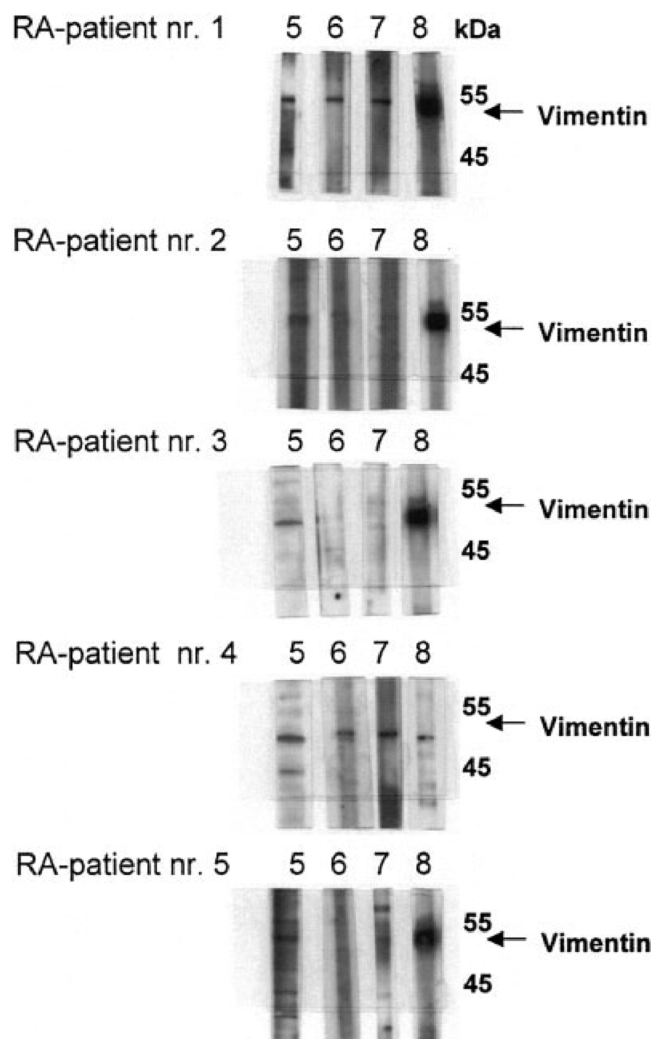

Figure 2. Vimentin isoforms purified from synovial fluid revealed identical recognition patterns as those obtained with sera from the same rheumatoid arthritis (RA) patients as shown in Figure 1B.

Table 1. Summary of peptides differing in either a citrullination or another modification of the vimentin sequence*

m/z				
Experimental	Theoretical	Start	End	Sequence
1808.9338	1808.8714	1	15	STRSVSSSSY(**Cit**)(**Cit**)MF**R**
1808.9338	1808.8714	1	15	ST(**Cit**)SVSSSSYR(**Cit**)MF**R**
1808.9338	1808.8714	1	15	ST(**Cit**)SVSSSSY(**Cit**)RMF**R**
2505.3303	2505.1905	4	27	SVSSSSYR(**Cit**)MFGGPGTASRPSSSR
2505.3303	2505.1905	4	27	SVSSSSY(**Cit**)RMFGGPGTASRPSSSR
1159.6271	1159.582	16	27	***G***PGTASRPSSSR
1845.9554	1845.8943	19	35	***T***AS(**Cit**)PSSS(**Cit**)SYVTTSTR
2415.229	2415.2268	36	58	TYSLGSALRPSTS(**Cit**)SLYASSPG**R**
2415.229	2415.2268	36	58	TYSLGSAL(**Cit**)PSTSRSLYASSPG**R**
2359.1616	2359.1894	36	58	TYSLGSAL(**Cit**)PSTS(**Cit**)SLYASSPG**R**
2359.1616	2359.1894	36	57	TYSLGSAL(**Cit**)PSTS(**Cit**)SLYASSP**R**
1598.8157	1598.7485	143	154	S(**Cit**)L(**Cit**)DLYEEEMR
1896.9469	1896.9126	143	157	S(**Cit**)LGDLYEEEMRELR
1997.0159	1996.9762	143	157	S(**Cit**)L(**Cit**)DLYEEEMRELR
2525.2405	2525.2888	78	99	LLQDSVDFSLADAINTEF**m**KNT**m**R
1615.8317	1615.8291	100	112	TNE**m**KVELQELND**m**R
1528.231	1528.172	378	389	HL(**Cit**)EYQDLLNVK
2144.971	2144.034	373	389	EEMA(**Cit**)HLREYQDLLNVK

* Peptides from human vimentin isoforms derived by tandem mass spectrometry contain citrullinated and mutated amino acid residues and were selected for presentation. Peptides were identified by correlation of uninterpreted tandem mass spectra to entries in Swiss-Prot/TrEMBL. Citrullinated residues, new tryptic cleavage sites, and mutations were localized by manual interpretation of the sequence-specific fragment ion. m/z = molecular size; start and end = start position and end position of the sequenced peptide excluding the first methionine amino acid residue; (**Cit**) = citrullination (Arg→Citr); **R** = mutation of a glycine residue in an arginine residue (G/C transversion, Gly→Arg); ***G*** or ***T*** = new tryptic cleavage site (N-terminal) caused by G/C transversion; (**Cit**) = citrullination (Arg→Citr) after mutation of a glycine residue in an arginine residue (G/C transversion, Gly→Arg); **m** = monomethylated amino acid.

time 6 hours to 2 days) according to the reported procedure (24). Trypan blue exclusion assay revealed that cell viability was not reduced by treatment with 1 μM H_2O_2 for 2 days, as compared with untreated fibroblasts (data not shown). As an important result, exposure to H_2O_2 strongly increased the expression of unusual vimentin isoforms identified by the established purification procedures and immunoblotting. Interestingly, no additional isoforms were observed in H_2O_2-treated cells compared with untreated cells (Figure 1A).

Anti-CCP–positive sera from 5 RA patients (3 with anti-CCP titers of 54, 120, and 542 units/ml and 2 with titers >1,600 units/ml) were used to investigate the different vimentin isoforms by immunoblotting (Figure 1B). Vimentin isoforms with pI 4.6–5.4 and 5.4–6.2 were recognized by only 2 of the RA sera, revealing a relatively low antibody sensitivity. In contrast, vimentin isoforms with pI 3.0–4.6 and 6.2–7.0 were recognized by all RA sera investigated. Sera from healthy volunteers were negative for any of the purified protein isoforms (data not shown). Therefore, the antigenic properties of vimentin were significantly influenced by oxidative stress.

Furthermore, anti–modified citrulline antibodies (Senshu antibodies) were used for the detection of citrulline-containing proteins (Figure 1C). Citrullinated proteins were detected in fractions with pI 3.0–4.6 and 6.2–7.0 after chemical modification of citrulline residues to form a ureido group adduct. Some bands were also stained with the antivimentin antibodies. These experiments suggest that the enhanced frequency and citrullination of unusual vimentin isoforms may be induced in fibroblast cell culture during exposure to H_2O_2.

Characterization of human vimentin isoforms as mutated and citrullinated antigens. To determine the nature of vimentin isoforms in RA, pooled human SF samples were used as a source of antigen preparations. According to the above-described experiments, separation was performed by size using fast-protein LC and chromatofocusing. In fact, semipurified vimentin isoforms revealed identical patterns of recognition by the above-noted antibodies in immunoblotting (Figure 2).

RP-HPLC provided the required purity and conserved autoantigenic properties of vimentin. After tryptic digestion of the major peak fraction, MALDI-TOF MS analysis and partially automatic Edman degradation

were performed. The sequences obtained were compared with the BLAST program in the Swiss-Prot database.

Forty-two peptides were sequenced, all of which mapped onto human vimentin (Swiss-Prot accession no. P08670). In total, 124 residues of nonredundant amino acid sequence were obtained, corresponding to the reported vimentin sequence.

Table 1 summarizes the peptides differing in either a citrullination or another modification of the vimentin sequence. Conversion of arginine to citrulline results in an increase in molecular mass of 0.984 daltons and in a modified acidic shift in the IEF migration due to loss of the positive charge of the side chain. Moreover, the pattern of peptides obtained on tryptic digestion will be altered, because the modified residues are refractory to trypsinolysis and result in another cleavage pattern, rather than carboxy-terminal arginine. Peptide sequences corresponding to the N-terminal domain of vimentin were susceptible to different modifications including transversion, monomethylation, and citrullination (Table 1). Summarized peptide sequences from the relevant vimentin isoforms (pI 3.0–4.6 and 6.2–7.0) showed no random citrullinations of the arginine residues, but instead, showed specificity for residues flanked by serine.

Seven peptides containing a carboxy-terminal arginine residue were analyzed and revealed a mutated vimentin sequence. In the wild-type vimentin sequence, a glycine residue is present instead of an arginine residue. This observation correlates with the known susceptible transversion of the guanine base in genomic DNA under oxidative stress. Interestingly, these newly incorporated arginine residues were only occasionally citrullinated. Therefore, antigenic properties of vimentin were substantially influenced by mutation in addition to citrullination. Additionally, the peptide sequences showed a C-terminal domain of vimentin that was highly resistant to modifications (Table 1), because citrullination was found only in the arginine residues between positions 370 and 390.

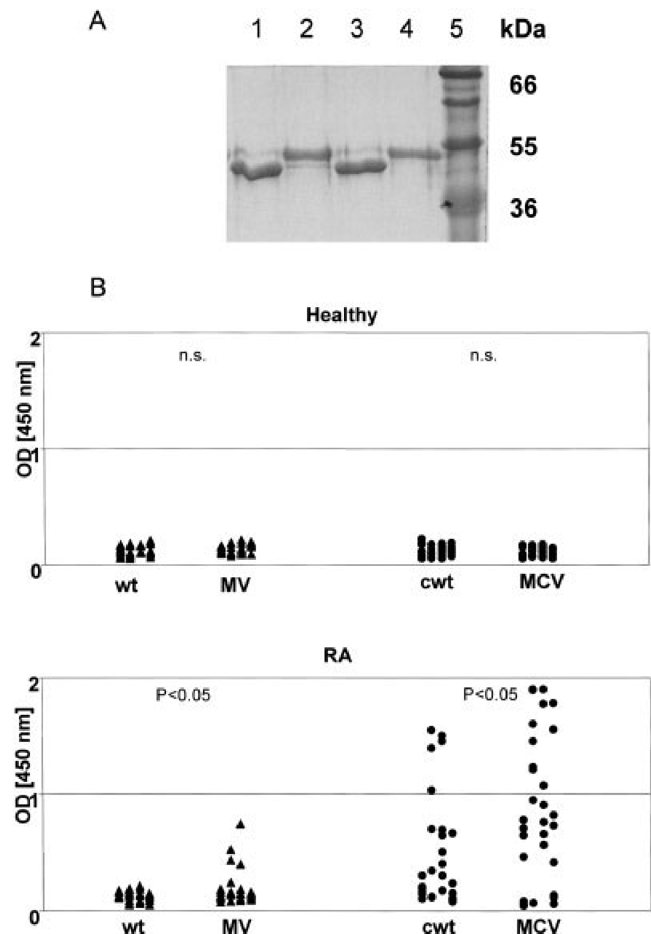

Figure 3. A, Coomassie blue staining of a sodium dodecyl sulfate–polyacrylamide gel of recombinant wild-type vimentin (lanes 1 and 2) and mutated vimentin (lanes 3 and 4), either without peptidyl arginine deiminase treatment (lanes 1 and 3) or in the citrullinated form (lanes 2 and 4). Lane 5 contains molecular weight markers. **B,** Scattergrams showing antibody reactivities against wild-type vimentin (wt) and mutated vimentin (MV) and against their in vitro citrullinated analogs (cwt and MCV, respectively), using sera from rheumatoid arthritis (RA) patients and healthy volunteers. OD = optical density; NS = not significant.

Expression of human MCV. To confirm a difference between the well-known wild-type vimentin and the mutated citrullinated antigen, the respective recombinant human proteins were expressed in *E coli*. Based on the reported sequence and the observed mutations, vimentin genes were amplified and cloned in a His-Tag expression vector. The human recombinant vimentins were purified by means of metal chelate affinity chromatography, size-exclusion chromatography, and, finally, anion ion-exchange chromatography. Purity of the recombinant antigens was verified by SDS-PAGE as a single-band protein of the expected size of 54 kd (Figure 3A). Finally, to combine the transcriptional and post-translational modifications of vimentins, the purified proteins were citrullinated with PAD in vitro. These modifications resulted in different migration properties of the citrullinated proteins in SDS-PAGE (Figure 3A). Importantly, enhanced antibody reactivity was observed against MCV compared with the citrullinated wild-type vimentin when sera from RA patients were used

(mean ± SD optical density 0.851 ± 0.626 versus 0.445 ± 0.456; $P < 0.05$) (Figure 3B).

Anti-MCV ELISA for the diagnosis and prognosis of RA. The presence of anti-MCV antibodies was investigated by a newly developed ELISA using the recombinant human MCV as antigen. CVs were calculated for each of 3 anti-MCV samples (obtained from patients with low, moderate, and high titers) based on the results of 24 determinations in a single run for intraassay precision. Run-to-run precision was calculated from the results of 6 different runs with 24 determinations. The intraassay CVs were 2.4%, 3.9%, and 3.2% for mean concentrations of 45.9 units/ml, 175.4 units/ml, and 762.0 units/ml, respectively, whereas the interassay CVs were 4.9%, 3.1%, and 3.2% for mean concentrations of 55.7 units/ml, 158.1 units/ml, and 744.0 units/ml, respectively.

Sera from 1,151 patients with RA were investigated in the anti-MCV antibody ELISA. Clinical and serologic data of the patients were obtained, including serum reactivities in a commercially available second-generation anti-CCP ELISA. As a result, the diagnostic specificity of the anti-MCV antibody ELISA was comparable with that of the anti-CCP assay (98% versus 96%). Interestingly, an improvement in sensitivity (82% versus 72%) was achieved by the anti-MCV antibody ELISA in the cohort analyzed (Table 2).

In order to correlate anti-MCV reactivity with RA disease activity as assessed by the Disease Activity Score in 28 joints (DAS28) (25), followup analyses of anti-MCV response were performed in 42 patients with RA over 427 different time points. Importantly, compared with the 16 patients with moderate RA (mean ± SD DAS28 2.72 ± 0.70), the 26 patients with active RA (mean ± SD DAS28 5.07 ± 0.77) expressed significantly higher antibody titers against MCV (mean ± SD 1,161 ± 1,341 units/ml versus 348 ± 494 units/ml; P = 0.0084 by t-test). In contrast, anti-CCP antibody reactivities were not significantly different between patients with active RA and those with moderate RA (464 ± 607 units/ml versus 549 ± 635 units/ml; P = 0.6697). Anti-MCV antibodies were significantly correlated (by Pearson's correlation coefficient) with disease activity in patients with RA (r = 0.5334, P = 0.0003), while anti-CCP antibodies failed to show a significant correlation in our cohort (r = 0.1289, P = 0.4159). A significant correlation was also seen between anti-MCV and anti-CCP antibody reactivities (r = 0.4113, P = 0.0068).

Table 2. Comparison of autoantibody reactivities against citrullinated antigens in 1,151 patients with rheumatoid arthritis*

	Anti-MCV	Anti-CCP
Concentration, mean ± SD units/ml	516.8 ± 612.4	537.0 ± 655.4
Positive patients, no.	950	831
Negative patients, no.	201	320
Sensitivity, %	82	72
Specificity, %	98	96

* Anti-MCV = anti–mutated citrullinated vimentin; anti-CCP = anti–cyclic citrullinated peptide.

Table 3. Anti–mutated citrullinated vimentin antibodies in 189 patients with SLE, 68 patients with primary SS, and 232 healthy donors*

	SLE patients	Primary SS patients	Healthy donors
Concentration, mean ± SD units/ml	19.6 ± 31.4	21.8 ± 16.2	9.1 ± 4.7
Positive patients, no. (%)	43 (22.8)	11 (16.2)	4 (1.7)
Negative patients, no. (%)	146 (77.2)	57 (83.8)	228 (98.3)

* SLE = systemic lupus erythematosus; SS = Sjögren's syndrome.

The prevalence of anti-MCV antibodies was analyzed in 232 healthy donors, 189 patients with SLE, and 68 patients with primary SS (Table 3). Remarkably, weak antibody reactivities were observed in 17.5% of patients with SLE and in 11.7% of patients with primary SS. Followup analyses of 10 patients with SLE and 10 patients with primary SS showed stable antibody titers against CCP and MCV within the reference range (data not shown).

DISCUSSION

In this study, we investigated the expression of vimentin in synovial sites under pathologic conditions. As an important result, we identified in RA patients an isoform of vimentin modified not only by citrullination, but also by mutation. Using this potentially relevant alternative antigen, we developed the first ELISA for the detection of autoantibodies against human MCV. Surprisingly, the antigenic properties of this molecule were strongly influenced by modification of the sequence. Compared with established markers, determination of anti-MCV antibodies provided the highest specificity and sensitivity for the diagnosis of RA in our cohort.

A decade ago, disease-specific autoantibody reactivity against the Sa antigen was described in patients with RA, and vimentin was subsequently identified as the target antigen, using antibodies against citrullinated epitopes. Therefore, antigen recognition by these RA-

specific autoantibodies is critically dependent on the presence of the unusual amino acid citrulline, formed by a posttranslational modification of arginine residues by the enzyme PAD. Interestingly, a predominant expression of PAD isoforms has been described in macrophages infiltrating the synovial tissue; moreover, vimentin was found to represent a predominant substrate of these enzymes, especially in activated and dying macrophages in patients with RA (26–28). It was shown that proteolysis of vimentin by caspases promotes apoptosis via a proapoptotic cleavage product (29,30). In fact, the occurrence of citrullinated vimentin in synovial membranes and its secretion by macrophages in response to growth factors and proinflammatory cytokines make it a relevant joint-associated autoantigen in the pathogenesis of RA (11,13). However, the prevalence of anti–citrullinated vimentin antibodies was relatively low, and this marker was not suitable to substantially improving the procedures for diagnosing RA.

Vimentin intermediate filaments are dynamic structures, and their flexible organization is important for the regulation of mechanical stress between chondrocytes and the surrounding matrix tissue. Furthermore, a role for vimentin was described in the regulation of T cell activation. As a posttranslational modification, citrullination of vimentin occurs under circumstances that are not unique to inflamed synovial tissue in RA (31). Recently, it was reported that different vimentin isoforms resulted from phosphorylation, degradation, or glutathionylation (32). In the present study, we identified transcriptional modifications of relevant epitopes of vimentin, providing a novel marker for the diagnosis and prognosis of RA.

Recently, it was shown that presentation of antigen variants to the immune system triggers the recognition of normally silent epitopes and can enhance the generation of adaptive immune responses to self (33). The assumption of existing isoforms of vimentin was based on the growing evidence of abundant microsatellite instability and suppression of mechanisms that limit genomic DNA damage in RA synovium (24). Moreover, overexpression of, and functional mutations in, key genes were shown for the rheumatoid synovium (e.g., for the p53 tumor suppressor gene) (34). As a relevant pathologic mechanism, it was assumed that persistent oxidative stress might be involved in DNA damage. In this context, an increased susceptibility to oxidative stress of the guanine base (G) must be taken into account due to the lowest oxidation potential. One typical lesion of G is 8-oxo-7,8-dihydro-guanine (8-oxoG), which can pair with A (for review, see refs. 35 and 36). This pairing may cause mutations of glycine residues to arginine residues. Although the number of G/C transversions is increased under exposure to oxygen radicals, the molecular basis of G/C transversions is not completely understood.

In the present investigation, we identified mutated glycine residues within the vimentin DNA caused by at least 1 single-nucleotide polymorphism. Moreover, it was shown that mutated vimentin is also citrullinated in SF of patients with RA. Our data indicate that citrullination by PAD is influenced by amino acid residues that flank arginine, resulting in a nonrandom modified protein. However, despite citrullination, mutation of vimentin represents an independent trigger of the antigenic properties of the antigen in RA.

Using the mutated and citrullinated recombinant human antigen vimentin for the diagnosis of RA in a standardized ELISA, we clearly documented a preserved high diagnostic specificity of the antigen. Of note, weak antibody reactivities against MCV were observed in a small subset of patients with SLE and primary SS. Therefore, the diagnostic significance of anti-MCV antibodies must be confirmed in a multicenter study. Importantly, the assay showed better sensitivity than the anti-CCP ELISA. Furthermore, anti-MCV titers correlated with disease activity in our cohort and, accordingly, may allow the stratification of RA. This raises the possibility that this novel marker may be suitable for both monitoring and diagnosing RA. However, even larger series will be necessary to fully appreciate the very important correlation between disease activity and anti-MCV levels, and such studies are currently being conducted by a European network. Our results strongly indicate that mutations and citrullinations of vimentin trigger the autoantibody response. The breakage of self tolerance to this antigen and the potential pathogenic significance of modified vimentin will be investigated in future studies.

AUTHOR CONTRIBUTIONS

Dr. Burmester had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study design. Bang, Egerer, Gauliard, Fredenhagen, Berg, Burmester.
Acquisition of data. Bang, Egerer, Gauliard, Lüthke, Fredenhagen, Berg, Burmester.
Analysis and interpretation of data. Bang, Egerer, Feist, Burmester.
Manuscript preparation. Bang, Egerer, Feist, Burmester.
Statistical analysis. Rudolph.

REFERENCES

1. Lee DM, Weinblatt ME. Rheumatoid arthritis. Lancet 2001;358: 903–11.

2. Firestein GS. Immunologic mechanisms in the pathogenesis of rheumatoid arthritis. J Clin Rheumatol 2005;11:39–44.
3. Van Boekel MA, Vossenaar ER, van den Hoogen FH, van Venrooij WJ. Autoantibody systems in rheumatoid arthritis: specificity, sensitivity and diagnostic value. Arthritis Res 2002;4:87–93.
4. Schellekens GA, de Jong BA, van den Hoogen FH, van de Putte LB, van Venrooij WJ. Citrulline is an essential constituent of antigenic determinants recognized by rheumatoid arthritis-specific autoantibodies. J Clin Invest 1998;101:273–81.
5. Van Gaalen F, Ioan-Facsinay A, Huizinga TW, Toes RE. The devil in the details: the emerging role of anticitrulline autoimmunity in rheumatoid arthritis. J Immunol 2005;175:5575–80.
6. Greiner A, Plischke H, Kellner H, Gruber R. Association of anti-cyclic citrullinated peptide antibodies, anti-citrullin antibodies, and IgM and IgA rheumatoid factors with serological parameters of disease activity in rheumatoid arthritis. Ann N Y Acad Sci 2005;1050:295–303.
7. Van der Helm-van Mil AH, Verpoort KN, Breedveld FC, Toes RE, Huizinga TW. Antibodies to citrullinated proteins and differences in clinical progression of rheumatoid arthritis. Arthritis Res Ther 2005;7:R949–58.
8. Despres N, Boire G, Lopez-Longo FJ, Menard HA. The Sa system: a novel antigen-antibody system specific for rheumatoid arthritis. J Rheumatol 1994;21:1027–33.
9. Escalona M, Lopez-Longo FJ, Gonzalez CM, Monteagudo I, Rodriguez-Mahou M, Grau R, et al. Anti-Sa sera from patients with rheumatoid arthritis contain at least 2 different subpopulations of anti-Sa antibodies. J Rheumatol 2002;29:2053–60.
10. Menard HA, Lapointe E, Rochdi MD, Zhou ZJ. Insights into rheumatoid arthritis derived from the Sa immune system. Arthritis Res 2000;2:429–32.
11. Vossenaar ER, Despres N, Lapointe E, van der Heijden A, Lora M, Senshu T, et al. Rheumatoid arthritis specific anti-Sa antibodies target citrullinated vimentin. Arthritis Res Ther 2004;6: R142–50.
12. Hill JA, Southwood S, Sette A, Jevnikar AM, Bell DA, Cairns E. Cutting edge: the conversion of arginine to citrulline allows for a high-affinity peptide interaction with the rheumatoid arthritis-associated HLA-DRB1*0401 MHC class II molecule. J Immunol 2003;171:538–41.
13. Mor-Vaknin N, Punturieri A, Sitwala K, Markovitz DM. Vimentin is secreted by activated macrophages. Nat Cell Biol 2003;5:59–63.
14. Chang L, Goldman RD. Intermediate filaments mediate cytoskeletal crosstalk. Nat Rev Mol Cell Biol 2004;5:601–13.
15. Yang X, Wang J, Liu C, Grizzle WE, Yu S, Zhang S, et al. Cleavage of p53-vimentin complex enhances tumor necrosis factor-related apoptosis-inducing ligand-mediated apoptosis of rheumatoid arthritis synovial fibroblasts. Am J Pathol 2005;167:705–19.
16. Boire G, Cossette P, de Brum-Fernandes AJ, Liang P, Niyonsenga T, Zhou ZJ, et al. Anti-Sa antibodies and antibodies against cyclic citrullinated peptide are not equivalent as predictors of severe outcomes in patients with recent-onset polyarthritis. Arthritis Res Ther 2005;7:R592–603.
17. Nijenhuis S, Zendman AJ, Vossenaar ER, Pruijn GJ, van Venrooij WJ. Autoantibodies to citrullinated proteins in rheumatoid arthritis: clinical performance and biochemical aspects of an RA-specific marker. Clin Chim Acta 2004;350:17–34.
18. Arnett FC, Edworthy SM, Bloch DA, McShane DJ, Fries JF, Cooper NS, et al. The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. Arthritis Rheum 1988;31:315–24.
19. Tan EM, Cohen AS, Fries JF, Masi AT, McShane DJ, Rothfield NF, et al. The 1982 revised criteria for the classification of systemic lupus erythematosus. Arthritis Rheum 1982;25:1271–7.
20. Vitali C, Bombardieri S, Jonsson R, Moutsopoulos HM, Alexander EL, Carsons SE, et al, and the European Study Group on Classification Criteria for Sjögren's Syndrome. Classification criteria for Sjögren's syndrome: a revised version of the European criteria proposed by the American-European Consensus Group. Ann Rheum Dis 2002;61:554–8.
21. Larsen A, Dale K, Eek M. Radiographic evaluation of rheumatoid arthritis and related conditions by standard reference films. Acta Radiol Diagn (Stockh) 1977;18:481–91.
22. Senshu T, Akiyama K, Kan S, Asaga H, Ishigami A, Manabe M. Detection of deiminated proteins in rat skin: probing with a monospecific antibody after modification of citrulline residues. J Invest Dermatol 1995;105:163–9.
23. Kahaly GJ, Bang H, Berg W, Dittmar M. Alpha-fodrin as a putative autoantigen in Graves' ophthalmopathy. Clin Exp Immunol 2005;140:166–72.
24. Lee SH, Chang DK, Goel A, Boland CR, Bugbee W, Boyle DL, et al. Microsatellite instability and suppressed DNA repair enzyme expression in rheumatoid arthritis. J Immunol 2003;170:2214–20.
25. Prevoo ML, van 't Hof MA, Kuper HH, van Leeuwen MA, van de Putte LB, van Riel PL. Modified disease activity scores that include twenty-eight–joint counts: development and validation in a prospective longitudinal study of patients with rheumatoid arthritis. Arthritis Rheum 1995;38:44–8.
26. Moisan E, Girard D. Cell surface expression of intermediate filament proteins vimentin and lamin B1 in human neutrophil spontaneous apoptosis. J Leukoc Biol 2006;79:489–98.
27. Asaga H, Yamada M, Senshu T. Selective deimination of vimentin in calcium ionophore-induced apoptosis of mouse peritoneal macrophages. Biochem Biophys Res Commun 1998;243:641–6.
28. Vossenaar ER, Radstake TR, van der Heijden A, van Mansum MA, Dieteren C, de Rooij DJ, et al. Expression and activity of citrullinating peptidylarginine deiminase enzymes in monocytes and macrophages. Ann Rheum Dis 2004;63:373–81.
29. Dinsdale D, Lee JC, Dewson G, Cohen GM, Peter ME. Intermediate filaments control the intracellular distribution of caspases during apoptosis. Am J Pathol 2004;164:395–407.
30. Byun Y, Chen F, Chang R, Trivedi M, Green KJ, Cryns VL. Caspase cleavage of vimentin disrupts intermediate filaments and promotes apoptosis. Cell Death Differ 2001;8:443–50.
31. Vossenaar ER, Smeets TJ, Kraan MC, Raats JM, van Venrooij WJ, Tak PP. The presence of citrullinated proteins is not specific for rheumatoid synovial tissue. Arthritis Rheum 2004;50:3485–94.
32. Fratelli M, Demol H, Puype M, Casagrande S, Eberini I, Salmona M, et al. Identification by redox proteomics of glutathionylated proteins in oxidatively stressed human T lymphocytes. Proc Natl Acad Sci U S A 2002;99:3505–10.
33. Engelhorn ME, Guevara-Patino JA, Noffz G, Hooper AT, Lou O, Gold JS, et al. Autoimmunity and tumor immunity induced by immune responses to mutations in self. Nat Med 2006;12:198–206.
34. Salvador G, Sanmarti R, Garcia-Peiro A, Rodriguez-Cros JR, Munoz-Gomez J, Canete JD. p53 expression in rheumatoid and psoriatic arthritis synovial tissue and association with joint damage. Ann Rheum Dis 2005;64:183–7.
35. Cooke MS, Evans MD, Dizdaroglu M, Lunec J. Oxidative DNA damage: mechanisms, mutation, and disease. FASEB J 2003;17: 1195–214.
36. Ames BN, Shigenaga MK, Hagen TM. Oxidants, antioxidants, and the degenerative diseases of aging. Proc Natl Acad Sci U S A 1993;90:7915–22.